Hydrophobic Pulmonary Surfactant Proteins SP-B and SP-C Induce Pore Formation in Planar Lipid Membranes: Evidence for Proteolipid Pores

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ABSTRACT Pulmonary surfactant is a complex mixture of lipids and specific surfactant proteins, including the hydrophobic proteins SP-B and SP-C, in charge of stabilizing the respiratory surface of mammalian lungs. The combined action of both proteins is responsible for the proper structure and dynamics of membrane arrays in the pulmonary surfactant network that covers the respiratory surface. In this study, we explore the possibility that proteins SP-B and SP-C induce the permeabilization of phospholipid membranes via pore formation. To this end, electrophysiological measurements have been carried out in planar lipid membranes prepared with different lipid/protein mixtures. Our main result is that channel-like structures are detected in the presence of SP-B, SP-C, or the native mixture of both proteins. Current traces show a high variety of conductance states (from pS to nS) that are dependent both on the lipid composition and the applied potential. We also show that the type of host lipid crucially determines the ionic selectivity of the observed pores: the anionic selectivity observed in zwitterionic membranes is inverted to cationic selectivity in the presence of negatively charged lipids. All those results suggest that SP-B and SP-C proteins promote the formation of proteolipid channels in which lipid molecules are functionally involved. We propose that proteolipidic membrane-permeabilizing structures may have an important role to tune ionic and lipidic flows through the pulmonary surfactant membrane network at the alveolar surfaces.

INTRODUCTION

Pulmonary surfactant is a membrane-based system found in all pulmonated animals, which plays a crucial role to facilitate respiratory mechanics, stabilizing the air-water alveolar interface against the physical forces that induce alveoli to collapse (1). Furthermore, different components in surfactant are also responsible for the first innate response that is required to maintain the lung interface free of pathogens (2). The lack, deficiency, or inactivation of this system is associated with severe respiratory disorders such as the neonatal respiratory distress syndrome in premature babies (3) or the pulmonary dysfunction associated with acute respiratory distress syndrome in cases of lung injury (4).

Surfactant is a complex mixture of lipids and proteins, consisting of around 90% of lipids by mass, mainly phospholipids. An essential part (40–50% of the total mixture) is saturated phosphatidylcholines including dipalmitoylphosphatidylcholine, which is the main component responsible for reducing the interfacial surface tension down to nearly zero (5). There are also unsaturated phosphatidylcholines, anionic phospholipids such as phosphatidylglycerol (PG) and phosphatidylinositol, and an important fraction of neutral lipids, mainly cholesterol, which can account for up to 5–10% of the total lipid amount. The remaining 8–10% of the total mixture is constituted by specific proteins (6), which can be classified into two families: the hydrophilic proteins SP-A and SP-D, primarily related to the innate immune response in the alveolar barrier, and the hydrophobic proteins SP-B and SP-C, essential for the biophysical activity of pulmonary surfactant. Indeed, the presence of these two proteins is absolutely necessary for an efficient interfacial adsorption, film stability, and respreading processes of surfactant along the continuous compression-expansion breathing cycles (1).

SP-B is the only surfactant protein strictly required for breathing. Indeed, the absence of SP-B is associated with a lethal respiratory failure (7). SP-B is a 79-residue polypeptide, found as a 19 kDa homodimer in surfactant membranes (8). It belongs to the saposin-like protein family and consists of five amphipatic α-helices connected by highly apolar loops, exhibiting a positive net charge of $+7e$, which yield a preferential interaction with anionic phospholipids (9,10). In vitro, it has been shown that SP-B induces aggregation, fusion, and lysis of phospholipid vesicles (11), which has been associated with in vivo activities such as lipid transfer between bilayers and monolayers (12) and all the membrane restructuration processes that are required during the metabolic cycle of pulmonary surfactant. SP-B, for instance, is known to be essential for the formation of lamellar bodies, the organelles through which type II pneumocytes assemble, store, and secrete pulmonary surfactant membranes (13). It is also required, together with SP-A, for membrane rearrangement processes converting the onion-like lamellar body particles into net-like structures called tubular myelin that spread along the alveolar aqueous subphase. Unraveled from tubular myelin or independently of it, membranes are reorganized into layers near the interface to facilitate the transfer of surface-active

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species from the subphase into the interface and vice versa (see review (14)).

The other hydrophobic surfactant protein, SP-C, is the only component of pulmonary surfactant that has been found exclusively in the lung tissue (15). It has been described to be a monomer of 4.5 kDa and 35 residues that forms a single α-helix, 37 Å long, whose central 23 Å segment is highly hydrophobic and spans the thickness of a dipalmitoylphosphatidylcholine bilayer with a transmembrane orientation (16). The SP-C N-terminal tail exhibits a higher hydrophilicity providing to the protein a net charge of $+2e$. Cysteins 5 and 6 in this N-terminal segment are palmitoylated, contributing to maintain a tight association of this segment of the protein with bilayers and interfacial films (17). SP-C is believed to promote and stabilize membrane-membrane and membrane-interface contacts (18,19), facilitating lipid exchange between bilayers or between bilayers and monolayers, with a likely participation of the N-terminal segment, which has been reported to induce perturbations in lipid packing (20). The combined action of both SP-B and SP-C is considered to be responsible for the proper arrangement of functional membrane arrays in surfactant complexes.

The ability of hydrophobic surfactant proteins to perturb lipid packing and dynamics in membranes has been well established. On the one hand, the sequence of SP-B is homologous to saposins, a family of membrane-associated proteins that include several recognized cytolysins and membrane pore-forming structures (21–23). Amoebapores and NK-lysin, belonging also to the saposin family, induce current fluctuations upon interaction with phospholipid bilayers (24). On the other hand, some evidences have been found about the possible dimerization of SP-C in both organic solvents and lipid membranes (25,26), which could precede formation of larger oligomers and the creation of temporary defects or pores in membranes. In a previous work, it was shown that both SP-B and SP-C, either together in their physiological mixture or separately, alter the structure and permeability of phospholipid membranes (27).

Interestingly, the ability of both SP-B and SP-C proteins to permeabilize artificial phospholipid membranes and different clinical surfactant preparations (28,29) has been linked to the existence of some channel-mediated transport (30). In this study, we get deeper insight into the characterization of these effects by studying the permeability of planar lipid membranes (PLM) supplemented with SP-B and/or SP-C, as a function of several factors including lipid composition and membrane potential. We show that SP-B and SP-C, both together and also individually, create pores that are not as well defined as typical proteinaceous ionic channels. Indeed, we find a high variety of conductance states (from pS to nS) that are dependent both on the lipid composition and the applied potential. We also demonstrate that the type of host lipid crucially determines the ionic selectivity of the observed pores. Our results suggest that SP-B and SP-C induce permeabilization changes in membranes via the formation of proteolipidic pores, where the proteins could act as scaffolds to induce formation of lipidic pores, with the lipid polar heads lining totally or partially the pore wall.

MATERIALS AND METHODS

Materials

Native hydrophobic surfactant proteins were obtained from porcine lungs as described previously (31). Organic extraction of purified surfactant and chromatographic separation in Sephadex LH-20 (Pharmacia, Uppsala, Sweden) allowed obtention of the hydrophobic protein fraction (PF) separately from the surfactant lipid fractions (32,33). A subsequent chromatographic step in LH-60 yielded purification of SP-B and SP-C (33). Protein solutions were routinely checked for purity by SDS-PAGE and quantified by amino acid analysis. 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DOPG), 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC), and 1,2-diphytanoyl-*sn*-glycero-3-phosphoserine (DPhPS) were purchased from Avanti Polar Lipids (Alabaster, AL). Total phospholipid concentration in the different samples was determined by phosphorus analysis upon phospholipid mineralization. Proteins and lipids were stored as chloroform/methanol 2:1 (v/v) solutions at −20°C.

PLM

The experiments were carried out by using a very low amount (0.01% for PF and 0.005% for isolated SP-B or SP-C, with respect to lipid weight) of hydrophobic surfactant proteins supplemented in phospholipid bilayers. Two monolayers were made from 5 mg/ml pentane solutions of DOPC or DOPC/DOPG (85:15, w/w) mixture onto subphases buffered with 5 mM Hepes pH 6.0 at different KCl concentrations at both sides of the Teflon chambers partitioned by a 15 μm thick Teflon film with 70–100 μm diameter orifices. Planar lipid bilayers were formed by monolayer apposition on the orifices previously treated with a 1% solution of hexadecane in pentane. Proteins (SP-B, SP-C, or the physiological mixture of both, PF) in chloroform/methanol were supplemented to the lipid solutions before monolayer formation only in one of the chamber sides, the *cis* side. Bilayer formation was directly detected and its thickness could be estimated by capacitance measurements. All the experiments were carried out at 25 ± 1°C.

Channel conductance measurements

An electric potential was applied using Ag/AgCl electrodes in 2 M KCl, 1.5% agarose bridges assembled within standard 250 ml pipette tips. Potential is defined as positive when it is higher at the side of the protein addition (the *cis* side), whereas the *trans* side is set to ground. An Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) in the voltage-clamp mode was used for measuring the current and applying potential. The membrane chamber and the head stage were isolated from external noise sources with a double metal screen (Amuneal Manufacturing, Philadelphia, PA).

Reversal potential measurements

Lipoprotein bilayers were formed with different salt concentrations at each side. A net ionic current appeared due to the concentration gradient, and it was manually set to zero by adjusting the applied potential. The potential needed to achieve zero current was then corrected by the liquid junction potential calculated from Henderson's equation to obtain the zero current potential or reversal potential (E_{rev}) (34).

RESULTS

Hydrophobic surfactant protein fraction in DOPC and DOPC/DOPG bilayers

Planar membranes formed by zwitterionic DOPC or by the negatively charged DOPC/DOPG (85:15, w/w) lipid mixture are impermeable to ions, at least in the range of applied voltages ($V < 150$ mV) and in the timescale of these experiments. However, when these lipids were supplemented with the total hydrophobic PF purified from porcine pulmonary surfactant complexes, we found a high pore-forming activity showing a wide variety of conductance states. Some of the recorded current traces are presented in Fig. 1 *A* for DOPC plus PF and in Fig. 1 *B* for DOPC/DOPG plus PF bilayers (protein/lipid ratio of 0.01% by weight, ~$1/10^5$ in molar ratio). The current versus time recordings do not show well-defined and highly reproducible unitary conductances characteristic of protein channels. Quite the opposite, current values and lifetimes of these states were very heterogeneous, even for the same applied voltage. Such variability allows several explanations: the coexistence of diverse channels in the bilayer, including a different number of protein monomers forming the pores and/or different protein conformations; the existence of one single kind of channel with a variety of substates, or the combination of both.

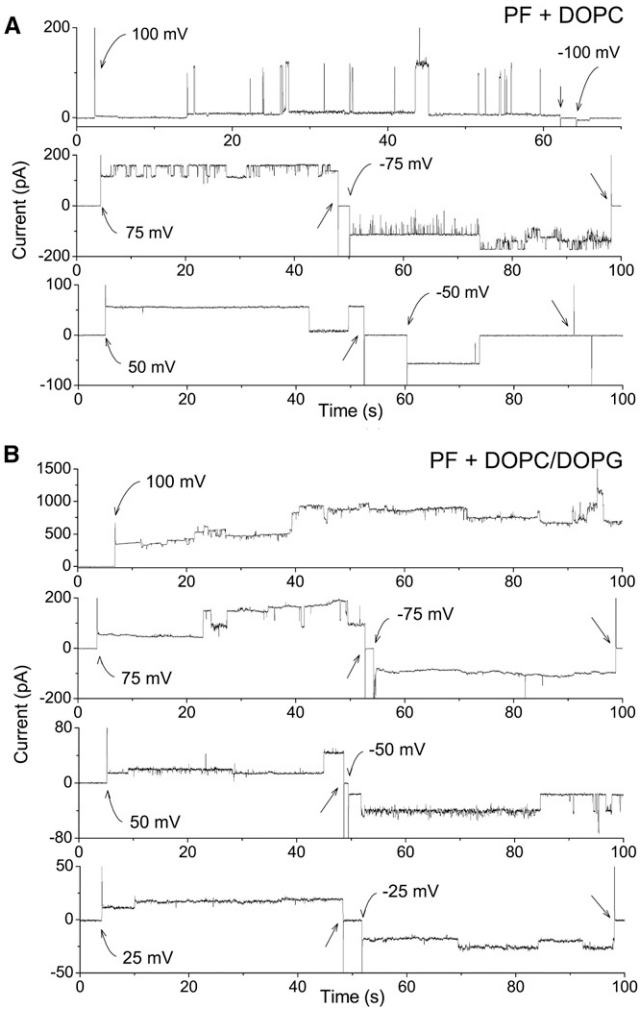

FIGURE 1 Effect of the hydrophobic surfactant protein fraction on the conductivity of zwitterionic and anionic phospholipid membranes. Several representative current versus time traces recorded for different applied voltages in DOPC (*A*) or DOPC/DOPG (85:15, w/w) (*B*); bilayers supplemented with the hydrophobic protein fraction of native surfactant, at a protein/lipid ratio of 0.01% by weight, in 5 mM Hepes 100 mM KCl solution, pH 5.5. Each trace corresponds to a different casted bilayer. Traces were subsequently filtered using a Bessel (8-pole) filter with a 50Hz −3dB cutoff frequency.

Histograms representing the conductance (G) levels of the current traces are shown in Fig. 2 *A*. Regardless of the lipid composition, a large dispersion in the measured conductance is observed. Under such conditions, we did not find a unitary or characteristic single-channel conductance. Small pores with conductance up to 100 pS were the most common, but we also found almost a continuous distribution of values ranging from tens of pS to several nS. We wondered whether the large values of conductance shown here in Fig. 1 correspond to multiple insertions of small pores rather than to individual wide pores. To discriminate between single- and multiple-channel conductances, a visual examination of the records shown in Fig. 1 was used to find the stepwise changes in each trace. Normalized histograms were then calculated from the conductance difference (ΔG) of the stepwise transitions as shown in Fig. 2 *B*. The plots of ΔG are almost identical to those of the absolute value of G, indicating that large values of ΔG are repeatedly obtained from single stepwise transitions. Because these large steps present long lifetimes without any small flickering (see the *bottom trace* in Fig. 1 *A*), it points to the existence of wide independent structures rather than to the simultaneous opening of the most abundant small structures. Apparently, the lipid composition does not have a dramatic impact on the histograms shown in Fig. 2 *A* and Fig. 2 *B*. In both zwitterionic and anionic lipid membranes, a somewhat continuous distribution of ΔG can be observed between tens of pS and several nS. However, a close inspection of the histograms reveals a subtle feature: the most frequent values of the stepwise changes in conductance are 93 pS in neutral DOPC and 55 pS in negatively charged DOPC/DOPG membranes.

Looking for additional insights about the pore structure, we also analyzed the effect of applied voltage on channel conductance G, as shown in Fig. 2 *C*. The obtained conductance values were classified as measured either at low (5–50 mV) or high voltages (75–100 mV). Note that we refer always to absolute values, as we observed no differences between applying positive or negative voltages. In the case of DOPC membranes high applied voltages increase significantly the probability of finding narrow pores, although the position of the maximum probability remains almost unchanged. This suggests that wide pores may

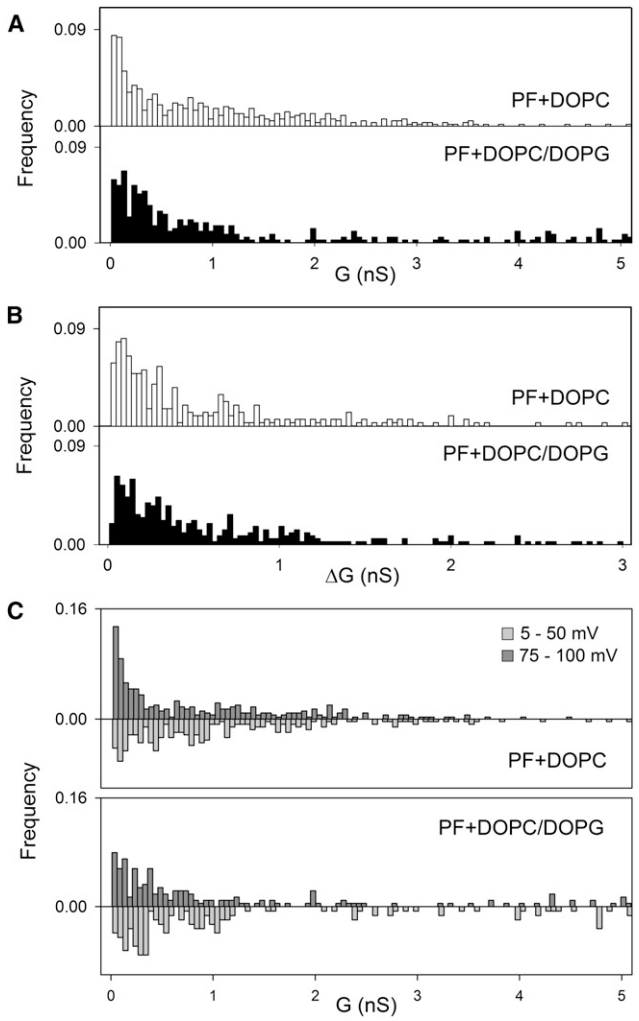

FIGURE 2 Quantitative analysis of the effect of surfactant proteins on the conductivity of zwitterionic and anionic phospholipid membranes. Normalized histograms representing all the conductance levels G (*A*) and conductance increments ΔG (*B*) recorded during the experiments with the hydrophobic protein fraction of native surfactant inserted in DOPC (*white bars*) or DOPC/DOPG (*black bars*) bilayers at a protein/lipid ratio of 0.01% by weight. Values of G and ΔG were calculated from average I and ΔI measurements divided by the correspondent applied voltage. In *C*, normalized histograms represent the recorded G-values classified by the voltage that was applied in each measurement: high voltages (75–100 mV, *dark gray*) and low voltages (5–50 mV in absolute value, *gray inverted bars*). These measurements were done in 5 mM Hepes 100 mM KCl solution, pH 5.5. Around 30 different bilayers were casted for each sample, obtaining from them around 400 G- and 200 ΔG-values in each case.

undergo a structural change or closure induced by voltage that does not affect the structure of the narrower ones. This could be due to squeezing out of solvent at high voltages, an effect already observed in membranes pretreated with hexadecane, as is the case here. In contrast, the effect of voltage in DOPC/DOPG membranes is just the opposite. The distribution of relative frequencies is not altered by voltage, although the position of the maximum is slightly shifted. The most frequent conductances are higher for low voltages (around 0.3 nS) than for high voltages (50 pS).

Hitherto, we have shown how the lipid composition determines not only the amplitude histograms of conductance but also their voltage dependence, suggesting a direct incorporation of lipid into the pore structure. Thus, the lipid charge is likely to be critical for the channel selectivity. To explore this possibility, we carried out selectivity experiments for the entire hydrophobic surfactant protein fraction supplemented to both DOPC and DOPC/DOPG bilayers, as shown in Fig. 3 *A*. In the presence of a salt gradient concentration between both sides of the bilayer and no applied voltage, there is a net flux of ions and hence an electric current appears. By measuring the applied voltage that is needed to zero the electric current (the so-called reversal potential, E_{rev}), it is possible to investigate the preferential passage of either positive or negative ions. In a zwitterionic phospholipid bilayer such as that formed by DOPC, the pores were found to be selective to anions (*upper part* of Fig. 3 *A*). This fact is consistent with the positive net charge of both SP-B and SP-C, and, consequently, of the whole membrane surface. It is interesting to stress that no significant differences were observed between adding the proteins to the *cis* or to the *trans* side of the experimental cell while keeping the same KCl concentration ratios (results not shown), which suggests that there is no charge asymmetry with respect to the membrane plane. Interestingly, the incorporation of only 15% of DOPG by total weight of phospholipids into DOPC bilayers causes a dramatic impact. Regardless of the electrolyte concentration ratio, all the measured reversal potentials were negative (*bottom part* of Fig. 3 *A*), yielding a cationic selectivity. A similar selectivity inversion has also been observed in DPhPC (zwitterionic) and DPhPC/DPhPS (negatively charged) bilayers (results not shown here), indicating that it is the charge of the lipid polar head that originates this phenomenon. The critical importance of the lipid charges was already shown in the work by Oelberg and Xu (30), who used negatively charged membranes and reported cationic selectivity, consistently with the results presented here.

The qualitative interpretation of Fig. 3 *A* can be complemented using the well-known Goldman-Hodgkin-Katz equation to calculate the permeability ratio $P = P_+/P_-$ (35). Thus, the pores in DOPC bilayers are around 2.0–5.5 times more selective for Cl^- than for K^+, whereas in DOPC/DOPG bilayers the pores were between 2 and 2.5 times more selective for K^+ than for Cl^-. Therefore, the pores formed by the physiological mixture of SP-B and SP-C appear to be somehow more selective in zwitterionic than in negatively charged bilayers, probably because negative charges of PG counteract or neutralize the electrostatic interactions between Cl^- ions and positive charges of the polar residues of the proteins.

It is also worth mentioning that the measured values of the reversal potential in Fig. 3 *A* display a considerable

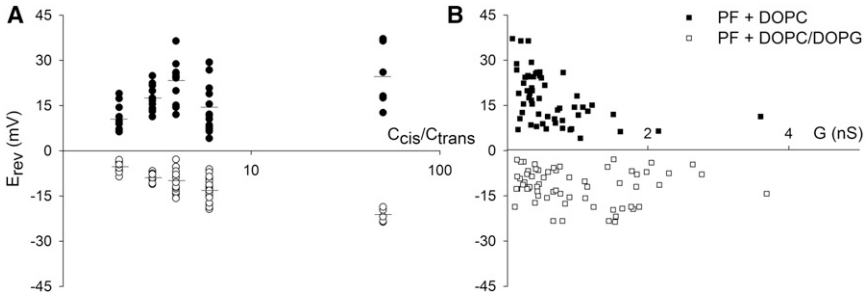

FIGURE 3 Ionic selectivity in zwitterionic and anionic phospholipid membranes containing the hydrophobic surfactant protein fraction. (*A*) Reversal potential versus KCl gradient concentration (50 mM trans side; 100, 150, 200, 300, and 2500 mM *cis* side) for DOPC (*solid circles*) or DOPC/DOPG 85:15 (*open circles*) bilayers supplemented with the hydrophobic protein fraction at a protein/lipid ratio of 0.01% by weight. Logarithmic scale has been used for the *x* axis. All the measured values have been plotted, and the black line represents the average of the measurements for each gradient concentration. (*B*) Reversal potential versus conductance for all the experiments performed with the hydrophobic protein fraction supplementing DOPC (*solid circles*) or DOPC/DOPG 85:15 (*open circles*) bilayers.

dispersion that is clearly beyond the experimental error. Such a scattered pattern is consistent with the existence of multiple independent pore structures as suggested by the data of Fig. 1. Note that the reversal potential of a collection of identical channels is independent of the actual number of them and should yield the same selectivity as a single unit (35). Therefore, if the variety of high conductance states found in Fig. 1 were actually originated by clusters of small structures, dispersion of reversal potential measurements would be within the experimental error.

We aimed to go a step further in the correlation between reversal potential (~selectivity) and pore conductance (~size); therefore, we calculated the conductance of the bilayer in each selectivity experiment, considering the full current-voltage (I-V) curve (note that when the I-V plot is linear, the slope gives the conductance and the intercept with V axis corresponds to the reversal potential). The correlation between E_{rev} and G can be seen in Fig. 3 *B*. Although the results are clearly scattered, a general trend could be observed, especially in the case of PF+DOPC (*solid symbols*): higher conductances correspond to lower reversal potentials, which means lower selectivity. This sounds reasonable, because narrow channels display a low conductance and a high discrimination because of the tight interaction between the permeating ions and the charges in the pore wall, whereas wider pores provide an easier and faster pathway at the price of losing selectivity (36). However, we should be cautious about this, because the scenario is likely to be much more complex: in different experiments we have found structures with a similar conductance but very different reversal potential.

Independent action of SP-B and SP-C in DOPC/DOPG bilayers

Having come this far, we can wonder whether the pore formation observed for the physiological SP-B/SP-C mixture is related to the sole action of one of the proteins or if alternatively, both of them are necessary. Experiments performed in planar lipid bilayers made of the DOPC/DOPG (85:15) mixture containing low amounts of either SP-B or SP-C (0.005% protein to lipid by weight, around $5\cdot10^{-6}$ for SP-B and 10^{-5} for SP-C in molar ratio) are shown in Fig. 4, *A* and *B*, respectively. Interestingly, each protein is able to induce pore formation, and both of them display a huge variety of conductance levels and current flickering. As in the case of the native mixture of SP-B and SP-C, a visual examination of the recorded traces shows large stepwise conductances for both proteins. This suggests that high conductance states are probably due to independent wide pores rather than to the concerted action of a cluster of small channels. Thus, in the top trace of Fig. 4 *A* (SP-B), we can observe a closure of around 2 nS and a subsequent opening of around 3 nS. In Fig. 4 *B* (SP-C) a conductance state of around 0.3 nS appears when applying 75 mV (*second trace from top*), and later on, two consecutive opening and closing events occur with stepwise conductances of 0.2 and 0.7 nS, respectively.

The histograms of conductance for each individual protein, shown in Fig. 5, resemble qualitatively the results found for the complete protein fraction in Fig. 2. At first sight, Fig. 5 *A* seems to indicate a slight difference between the two proteins. SP-B supplemented in DOPC/DOPG bilayers showed a wide range of conductances; being the most commonly recorded around 40 pS. In contrast, the most frequent conductance found for SP-C was around 0.3 nS. However, a more careful analysis considering the stepwise conductance (ΔG) distributions (see Fig. 5 *B*) rules out this possibility and yields similar conductance levels for both proteins. The most frequent recordings in Fig. 5 *B* were found to be 0.21 nS for SP-B and 0.16 nS for SP-C in DOPC/DOPG bilayers. This means that the most frequent stepwise conductance is larger for each protein individually than for the native mixture of both proteins (0.05 nS in Fig. 2 *B*). We also analyzed the effect of the applied potential in the independent SP-B and SP-C conductances measured in DOPC/DOPG bilayers, as shown in Fig. 5 *C*. In the case of SP-B the conductance distribution seems to be independent of applied voltage: there is no significant difference between low (5–50 mV) and high voltages (75–100 mV). In contrast, the normalized histograms for SP-C display a different scenario: the most common conductances at low applied voltages become much less frequent at higher potentials yielding a broader and less clear peak distribution.

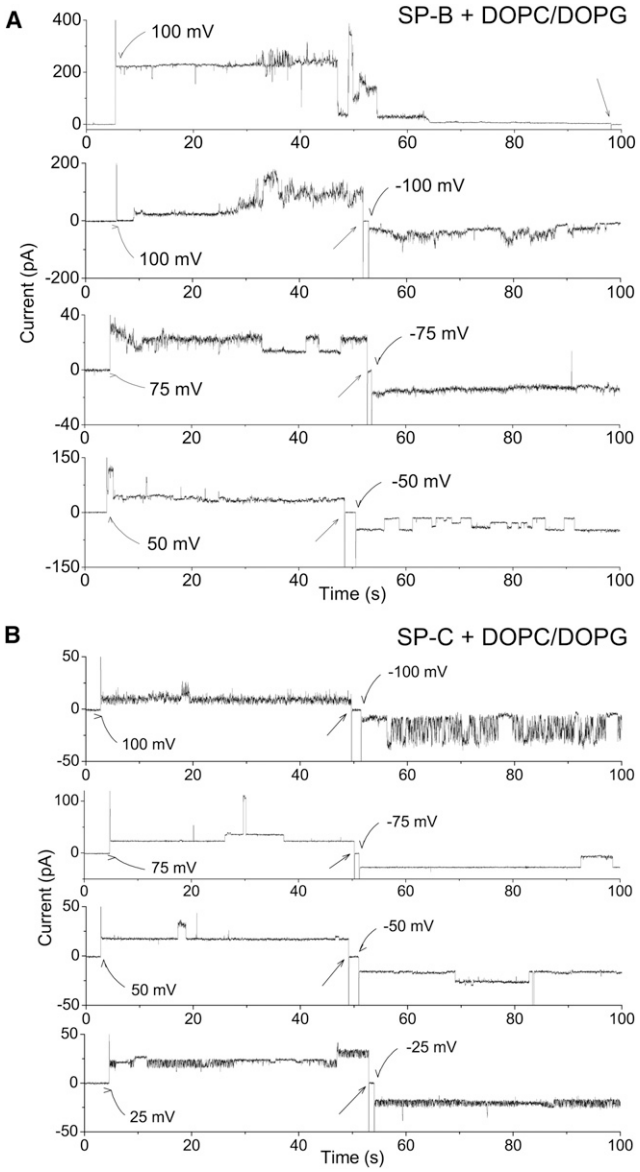

FIGURE 4 Effect of isolated protein SP-B or SP-C on the conductivity of anionic phospholipid membranes. Plotted are several representative current versus time traces recorded for different applied voltages in DOPC/DOPG 85:15 bilayers supplemented with SP-B (*A*) or SP-C (*B*) at a protein/lipid ratio of 0.005% by weight of phospholipids (around $5\cdot10^{-6}$ for SP-B and 10^{-5} for SP-C in molar ratio), in 5 mM Hepes 100 mM KCl solution, pH 5.5. Each trace corresponds to a different casted bilayer. Traces were subsequently filtered using a Bessel (8-pole) filter with a 50Hz −3dB cutoff frequency.

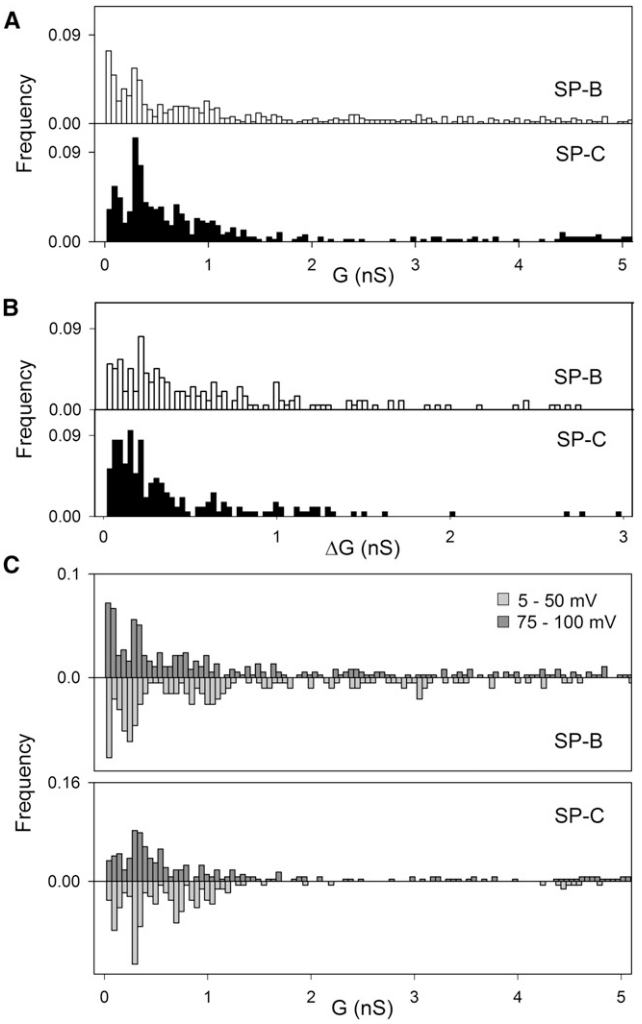

FIGURE 5 Quantitative analysis of the effect of isolated protein SP-B or SP-C on the conductivity of anionic phospholipid membranes. Normalized histograms representing all the conductance levels G (*A*) and conductance increments ΔG (*B*) recorded during the experiments with DOPC/DOPG 85:15 bilayers supplemented with SP-B (*white bars*) or SP-C (*black bars*) at a protein/lipid ratio of 0.005% by weight. Values of G and ΔG were calculated from average I and ΔI measurements divided by the correspondent applied voltage. In *C*, normalized histograms represent the recorded G-values classified by the voltage that was applied in each measurement: high voltages (75–100 mV, *dark gray*) and low voltages (5–50 mV in absolute value, *gray inverted bars*). These measurements were done in 5 mM Hepes 100 mM KCl solution, pH 5.5. Around 20 different bilayers were casted for each sample, obtaining from them around 500 G- and 200 ΔG-values in each case.

Reversal potential experiments in Fig. 6, *A* and *C*, show that both SP-B and SP-C form cation selective pores in DOPC/DOPG bilayers with a preference for K^+ over Cl^-. The corresponding permeability ratios P_+/P_- are in the range 1.9–2.6 for SP-B and 2.0–2.8 for SP-C. Because the selectivity of SP-B and SP-C pores is so similar despite the difference in the net charge of the proteins (+7 versus +2) suggests that the selectivity is mostly regulated by the lipid charge. Indeed, when selectivity experiments were done in zwitterionic DOPC bilayers (experiments not shown), pores formed by SP-B showed a preference for anions over cations, in agreement with the net charge of the protein (+7), whereas SP-C, in contrast, did not show a well-defined selectivity. Although average values would correspond to nonselective pores, we recorded small values of E_{rev} that were either positive or negative. This observation could occur because the net charge of the protein is still positive but considerably less important (+2) so that the

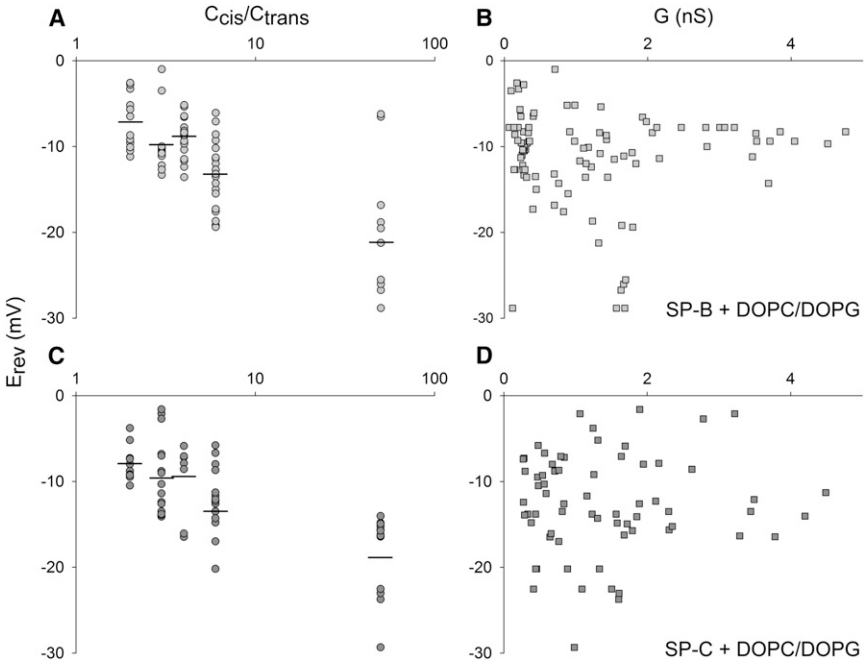

FIGURE 6 Ionic selectivity in anionic phospholipid membranes containing isolated protein SP-B or SP-C. (*A* and *C*) Reversal potential versus KCl gradient concentration (50 mM *trans* side; 100, 150, 200, 300, and 2500 mM *cis* side) for DOPC/DOPG 85:15 bilayers supplemented with SP-B (*A*) or SP-C (*C*) at a protein/lipid ratio of 0.005% by weight. Logarithmic scale has been used for the *x* axis. All the measured values have been plotted, and the black line represents the average of the measurements for each gradient concentration. (*B* and *D*) Reversal potential versus conductance for all the experiments performed with SP-B (*B*) or SP-C (*D*) supplementing DOPC/DOPG 85:15 bilayers.

experimental error is comparable to the absolute value of the reversal potential.

The relationship between pore conductance and reversal potential is shown for SP-B and SP-C in Fig. 6, *B* and *D*, respectively. The points are considerably scattered, therefore a possible correlation between pore size and selectivity is not as evident as in the native mixture of SP-B and SP-C shown in Fig. 3 *B*.

DISCUSSION

Numerous studies have reported the ability of hydrophobic surfactant proteins to alter the permeability of phospholipid membranes to polar solutes (8,11,14,16,27,28). This property has been usually linked to their properties to perturb lipid packing in membranes, required to facilitate the transfer of surface active species between surfactant membranes and the air-liquid interface. However, and beyond large membrane restructuration, it was recently shown that the mere presence of SP-B, SP-C, or both proteins together, make phospholipid membranes intrinsically permeable to polar, even charged, molecules (27). In this study, we have shown that SP-B and SP-C, both together and also individually, create an enormous diversity of defects or pores in phospholipid membranes, with no characteristic size or conformation in the experimental conditions tested here. Indeed, we have found a high variety of conductance states (from pS to nS) that are dependent both on the lipid composition and the applied potential. We have also shown that the type of host lipid crucially determines the ionic selectivity of the observed pores. These results suggest that SP-B and SP-C create pores not as well defined as typical proteinaceous ionic channels, but promote the local breakdown of the membrane bilayer structure creating pores with a surface formed partially or totally by polar lipid groups. SP-B belongs to the saposin-like family of proteins, which includes several members with detergent-like activity and true pore-forming toxins. This study reveals that the ability of SP-B to alter the permeability barrier of surfactant membranes could be not just the consequence of transient detergent-like local perturbations but of the assembly of long-living protein-lipid pore structures. A preliminary study had already proposed that a clinical surfactant preparation, with poorly defined lipid and protein composition, could contain ionic pores (30), although the unitary single-channel conductances of around 50 pS detected in those experiments were far from the large dispersion of small and wide pores found in our experiments with well-defined surfactant proteolipid membranes.

Each of the two proteins, SP-B and SP-C, seem able to produce a wide range of different conductances. A similar behavior has been observed in the pores formed by certain antibiotic peptides, such as colicin, which present two different conformations that are reflected in the recordings as two kinds of substates, a closed state with low conductance and an open state with high conductance (~0.05 nS and ~0.3–0.4 nS, respectively for colicin E1 in DPhPC bilayers) (37). In the case of SP-B and SP-C, there is no such sharp distinction between substates (see Fig. 2), perhaps because the possible conformations that these proteins adopted upon pore formation are more diverse. This may include the presence of different possible oligomerization states (38). The fact that, for the most frequent stepwise conductance, each protein yields individually larger values

than those found for the native mixture of both proteins (*0.05 nS* in Fig. 2 *B*) led us to hypothesize that in the entire protein fraction, SP-B might complex with SP-C to form a combined channel oligomer with average smaller aperture. The combined action of SP-B and SP-C could therefore somehow modulate the permeability of surfactant membranes and their ability to control transmembrane movement of charged species.

Several evidences in the current study support the concept that phospholipids take part of the surfactant protein-promoted permeabilizating structures. Although we have found that the full surfactant protein fraction PF introduces a continuous distribution of ΔG between tens of pS and several nS in both zwitterionic and anionic lipid membranes, the most frequent values of the stepwise changes in conductance are larger in neutral DOPC (93 pS) than in negatively charged DOPC/DOPG (55 pS). In principle, the negatively charged lipid headgroups in the vicinity of the channel would be expected to accumulate positive ions in excess of their bulk concentration, increasing then the pore conductance. Indeed, this is the observed effect in model protein channels like gramicidin A, alamethicin, or the bacterial porin OmpF (39–41). However, no such clear trend has been observed in proteolipidic pores. Malev et al. (42) reported that negatively charged lipids decreased the conductance of the syringomycin E channel at low electrolyte concentration and they had no clear effect in concentrated solutions. They suggested that the screening of the positive charges of the peptide and the lipid negative charges worked in opposite directions, partially compensating each other. We speculate that this could be also the case here. The negative charges of the DOPG lipid could balance or even overcompensate the effect of positive charges of both SP-B and SP-C proteins.

The fact that the ionic selectivity is inverted in the presence of negatively charged phospholipids compared with the behavior of the simultaneous presence of SP-B and SP-C in zwitterionic membranes, also argues in favor of a proteolipidic channel structure. Surfactant proteins might therefore promote or stabilize the opening of lipid-coated holes in phospholipid membranes, in the line of the so-called toroidal pores induced by other membrane-active peptides (43,44). Still, it remains to be demonstrated that the lipid molecules do actually line the protein-promoted membrane pores. It is also conceivable that the change in lipid selectivity promoted by acidic phospholipids could be alternatively due to a completely different organization of protein oligomers into the membrane. However, the high hydrophobicity of these proteins and their reported ability to facilitate rapid diffusion of lipids within surfactant membrane complexes (27), leads us to believe that the lipids must be lining the pores, at least partially. Different protein oligomers, either combining or not different proteins, could result in a range of different conductances such as those revealed by our experiments. The variety of conductive states could also represent a highly dynamic behavior, in which association/dissociation processes, involving or not protein homo- or heterooligomerization, could lead to fluctuations in membrane permeability. It has been reported that surfactant proteins SP-B and SP-C exhibit some affinity for negatively charged phospholipid species (9,10), with some controversy with respect to the actual specificity toward PG (45, 46), an essential phospholipid in surfactant activity. Affinity for anionic lipids is modulated by the ionic strength (9,10). It is therefore possible that the different ionic media used to analyze ion selectivity promote changes in lipid-protein interactions and in the oligomerization levels and conformational states of the proteins, all contributing to the complex picture observed in terms of multiple coexisting conductance situations. The conductive states that are the most relevant under physiological conditions remain to be determined.

We are aware that our results cannot be easily extrapolated to the biological system. It is evident that we are not taking into consideration the complex lipid composition in native surfactant membranes, and experiments have not been done at the physiological temperature. However, it is meaningful that these two small lipoproteins, isolated from its natural environment, could create such a significant change in a simple model membrane such as the one studied here. It had already been observed that the presence of SP-B and/or SP-C also increases dramatically the permeability of membranes made of the full lipid fraction of native surfactant (27). We have tried to prepare PLMs using the full lipid fraction of surfactant, but these membranes had a highly fluctuating character, with complex basal conductance states even in the absence of proteins (not shown), and were extremely unstable. This indicates that surfactant membranes with full compositional complexity could still be highly permeable but much more dynamic than the simplified models tested here.

The results we present here open, in our opinion, further interesting questions about the proteins themselves, which merit further research. For instance, it is intriguing how SP-C, with its extremely hydrophobic transmembrane helix, could affect the bilayer integrity to open aqueous pores. We speculate that a potentially important role may be played by the polar N-terminal segment of the protein, a region with the intrinsic potential to interact with and permeabilize phospholipid membranes (29,47). SP-B has been involved in modulating the mechanical properties of pulmonary surfactant membranes and films (48,49). A question of interest is how much the alteration of membrane geometry that leads to SP-B-promoted membrane permeabilization sustains the effect of the protein to stabilize multilayered films against mechanical perturbations. Finally, the SP-B and SP-C membrane poration activities reported here are highly indicative of the existence of high-order oligomerized states for the two proteins in membranes, something that has been previously proposed (38,50) but whose correlation with

the role of the proteins in surfactant is still not properly understood.

With respect to the biological significance of the existence of pores in surfactant membranes, we speculate that it could be related to the necessity of a continuous aqueous medium in the respiratory subphase to make it completely accessible for all the soluble molecules involved in innate defense (collectins, defensins are all hydrophilic molecules) and other processes in the lung interface (51). A highly permeable character of the membrane network formed by surfactant could be also important for a proper equilibrium of fluid and ions along the whole alveolar subphase. Permeability to polar molecules could be an intrinsic feature of what has been proposed as a potential bicontinuous surfactant membrane phase (14), evolutionarily optimized to permit simultaneously rapid diffusion of hydrophobic surface active species toward the respiratory interface and soluble molecules through the alveolar hypophase.

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